Isolating of a putative glyceraldehyde-3 phosphate dehydrogenase (GAPDH) from Calophyllum brasiliense, an important tropical forest tree

By C. M. PATREZE^{1),*)}, D. B. FELIX²⁾, F. R. SCARANO³⁾ and M. ALVES-FERREIRA²⁾

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Abstract

Calophyllum brasiliense Cambess. has characteristics that made it an excellent candidate model for ecogenomics in rain forest trees such as widespread natural occurrence and geographical patterns of adaptive genetic variation. Besides, it is also becoming a popular species for reforestation efforts in Brazil. Although, very little is known about its genetic diversity and the molecular mechanisms involved genetic adaptation traits. The first difficulty in launching genetic studies in a wild wood species is the lack of an optimized protocol for RNA and DNA isolation. In this work we built the essential framework for molecular genetics research with C. brasiliense comparing four distinct methods of RNA extraction from of three different tissues: leaves, stems and roots. We also were successful in the isolation of genomic DNA by an optimized CTAB method. Finally, degenerated oligonucleotide primers were designed for isolating of the glyceraldehyde-3 phosphate dehydrogenase (GAPDH) gene of C. brasiliense from the corresponding gene in closely related species. This gene is commonly used in plants as reference in expression gene analysis by Reverse Transcription Polymerase Chain Reaction (RT-PCR). Primers for RT-PCR were designed based on partial sequence obtained using degenerate primers designed. The optimized methods of RNA and DNA extraction combined with the identification, isolation and specific primer design for RT-PCR of a traditional Reference Gene provide the essential framework for molecular genetics research with C. brasiliense.

Key words: Calophyllum brasiliense; gene expression; nucleic acid extraction; ecogenomics.

Introduction

Calophyllum brasiliense Cambess. (Clusiaceae) is a hardwood tree claimed to be an alternative to mahogany (CARVALHO, 1994), which is almost exclusive to floodprone sites throughout a large geographic range in the neotropics (OLIVEIRA-FILHO and RATTER, 1995). For instance, in Amazon flooded forests this large tree is subjected to six months of deep river flooding and eventual submersion (e.g., KING, 2003), whereas in the swamp forests of southeast Brazil it undergoes almost permanent root flooding due to water table rises (SCARA-NO *et al.*, 1997; DUARTE *et al.*, 2005). Moreover, there is evidence that even within a small geographic range ecological behavior can be broadly variable among individuals of *Calophyllum brasiliense*. For example, FISCHER and DOS SANTOS (2001) have found differences in demography, sexual expression, phenology and flower and fruit production for this species among three contiguous habitats found in the lowlands within the domain of the Brazilian Atlantic rain forest of southeast Brazilunflooded forest, freshwater-flooded forest and mangrove in an old-growth estuarine forest.

Thus, geographical patterns of adaptive genetic variation are expected for C. brasiliense that turns it into an excellent candidate for studying the molecular mechanisms involved in genetic adaptation traits, especially those related to oxygen deprivation in consequence of flooding. The unveiling of the mechanisms of adaptation through the incorporation of genomic tools, i.e. ecogenomics, has poplars (Populus spp.) and conifers (e.g. Pinus spp., Pseudotsuga menziesii and Cryptomeria japonica) (FEDER and MITCHELL-OLDS, 2003; NEALE and SAVOLAINEN, 2004), as typical model species in forestry. While trees are ideal organisms to unveil the molecular basis of population adaptive divergence in nature (GONZÁLEZ-MARTÍNEZ et al., 2006), there are still no model species from the rain forests, and we expect that C. brasiliense could fill this gap. Moreover, its current use in commercial (HOLL, 1998) and ecologically-oriented (BOZELLI et al., 2000) reforestation efforts in Brazil highlight the importance of this tropical tree.

A first step to genetic analysis is the isolation of plant RNA and DNA, which are particularly challenging tasks, in particular from uncultivated or woody species. One of the major issues with plant nucleic acid isolation is the presence of problematic biomolecules, such as polyphenolic compounds and polysaccharides that interfere in the quantification and also trigger degradation of nucleic acids. Thus, extracting RNA and DNA from plant tissues often requires adaptation of existing protocols or new procedures. This is particularly true for woody species as it was shown for species of *Pinus* (CHANG *et al.*, 1993; CLAROS and CÁNOVAS, 1998), *Malus* (GASIC *et al.*, 2004), *Prunus* (MEISEL *et al.*, 2005), *Litchi* (WANG *et al.*, 2008), among others.

The gene expression analysis for species whose genomes are not sequenced is a critical challenge. The advent of the Reverse Transcription Polymerase Chain Reaction (RT-PCR) technique for the measurement of gene expression has allowed an accurate determination of the expression levels of target genes when compared

¹) Department of Botany, Federal University of Rio de Janeiro State, Centro de Ciências Biológicas e da Saúde, 22290240 Rio de Janeiro, Brazil.

²) Department of Genetics, Federal University of Rio de Janeiro, Centro de Ciências da Saúde, 21949900 Rio de Janeiro, Brazil.

³) Department of Ecology, Federal University of Rio de Janeiro, CCS, IB, BP 68020, 21941-970 Rio de Janeiro, Brazil.

^{*)} Corresponding author: CAMILA MAISTRO PATREZE. Universidade Federal do Estado do Rio de Janeiro, Instituto de Biociências, Departamento de Botânica. Av. Pasteur, n° 458, Urca, sala 409, 22290240 - Rio de Janeiro, RJ - Brasil. Tel. +552122445681, Fax +552122756059. E-Mail: <u>cpatreze@hotmail.com</u>.

to Northern blot analysis (DEAN et al., 2002). This former technique is time-consuming and requires a large quantity of RNA. Semi-quantitative gene expression data are often normalized to the expression levels of control or so-called "housekeeping" genes that show remain constant gene expression levels in distinct tissues under investigation. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a classical glycolytic enzyme, is involved in cellular energy production and has important housekeeping functions. The expression of GAPDH has been proved to be highly stable in different organs and experimental condition of several plant species as Saccharum sp. (ISKANDAR et al., 2004), Vitis vinifera (REID et al., 2006); Arabidopsis thaliana and Thlaspi caerulescens (KÜPPER et al., 2007); Lolium perenne L. (MARTIN et al., 2008); Coffee arabica (CRUZ et al., 2009); Gossypium hirsutum (ARTICO et al., 2010). In spite of its growing importance, there are not sequences of C. brasiliense available in any public database up to now by our knowledge. We consider that GAPDH gene might be also a good reference gene for gene expression analysis, therefore we focus on its isolation and characterization in C. brasiliense.

The use of degenerate oligonucleotide primers is one of the established methods employed to isolate genes from non-model plants, such as *C. brasiliense* (Löffert *et al.*, 1998). Recently, the use of degenerate primers was successful employed to isolated genes from some species such as *Elaeis guineensis* (OMAR *et al.*, 2008); *Trifolium repens* L. (TANG *et al.*, 2010); *Vitis amurensis* (KISELEV and DUBROVINA, 2010). MARTIN *et al.* (2008) isolated nine candidates to housekeeping gene, including *GAPDH*, from *Lolium perenne* L. using the degenerated primers methodology. However, none gene from *C. brasiliense* has been described using this method up to now.

In this work we ascertained the best method for RNA isolation from different *C. brasiliense* tissues after testing four distinct methods. We also described an adapted protocol for DNA isolating from *C. brasiliense* based on a method developed for DNA isolation from eucalyptus ssp. (FERREIRA and GRATTAPAGLIA, 1995). The methods described here are efficient for RNA and DNA extraction from different tissues and they provide a good quality material for enzymatic assays, such as cDNA synthesis or restriction enzyme reactions. In addition, we isolated the *C. brasiliense GAPDH* gene, a traditional reference gene, a crucial step for performing gene expression analysis by RT-PCR or real-time PCR.

Materials and Methods

Plant materials

Seedlings of *C. brasiliense* were grown in a greenhouse at a thermo period of $25/20 \pm 2$ °C and natural conditions of luminosity in the Federal University of Rio de Janeiro, Brazil. Leaves, stems and roots of two plants, four months old, were collected, immediately frozen in liquid nitrogen and subsequently ground into fine powders using a mortar and a pestle in liquid nitrogen. The resulting tissue powder was stored at -80 °C until use.

DNA isolation

The total DNA from leaves, stems and roots were extracted using an optimized method adapted from a protocol previously described by FERREIRA and GRATTA-PAGLIA (1995).

The plant tissue was ground with mortar and pestle to a fine powder in the presence of liquid nitrogen. The powder (300 mg) was transferred to a microtube with 900 µL of extraction buffer containing all reagents described by FERREIRA and GRATTAPAGLIA (1995) at the same concentrations, except for CTAB (hexadecyltrimethylammonium bromide) and 2-β-mercaptoethanol, which the concentration were increased to 2 and 1%, respectively. Each sample was mixed by vortexing the tube, which was then incubated at 65°C in a water-bath for 30–60 min with occasional shaking after each 10–15 min. Chloroform/isoamylalcohol (24:1, v/v) extractions were performed and the DNA was precipitated by addition of 0.1 volume of CTAB buffer (10% CTAB, 1.4 M NaCl) and homogenizing by inversion for 5 min, followed by addition of 2/3 volumes of isopropanol and incubation at -20 °C overnight. The sample was then centrifuged at 13,000 rpm for 20 min at 4°C. The pellet was washed two times with 1 mL of cold ethanol 70%for 3-5 min and once with 1 mL 95% of cold ethanol for 2-3 min. The DNA pellet was air-dried for 30 min. The pellet was ressuspended in buffer TE (10mM Tris-HCl (pH 8.0), 1 mM EDTA) for 30 min at room temperature. RNAse (10 µg/mL) digestion was performed by incubation at 37°C for 30 min. The quality and yield of total DNA extracted from different tissues were assessed by gel analysis and NanoDrop, model ND-1000 spectrophotometer (NanoDrop Technologies; Wilmington, DE).

Four restriction enzymes were used to check the DNA quality for enzymatic manipulation: PstI (octa-cutter) (Invitrogen), EcoRI (Invitrogen), HindIII (hexa-cutter) (Invitrogen) and HinfI (tetra-cutter) (Invitrogen). The enzymes were selected according to their frequency of sites in genomic DNA, an important characteristic for assessment of diversity using DNA-based techniques such as AFLP. Digestions were performed according to the manufacturer's recommendations for 2 hours at 37°C. One microgram of each DNA sample extracted of C. brasiliense leaf, stem and root were used in the assays.

$RNA \ isolation \ protocols$

One-hundred milligrams of fresh plant tissues (leaves, stems or roots) were used in four different protocols. The extraction procedures – (1) Trizol[®] (Invitrogen); (2) RNeasy[®] Plant Mini Kit (Qiagen) and (3) – Invisorb[®] Spin Plant RNA Mini Kit (Invitek) were performed following the manufacturing's instructions. For all procedures, two biological replicates were prepared. A fourth method adapted from WANG *et al.* (2008) (4) was tested using 1 g of fresh tissue. The method reported by WANG and collaborators uses ethylenediaminetetraacetic acid (EDTA), N-lauroyl sarcosine sodium (LSS) in combination with cetyltrimethylammonium bromide (CTAB). Sodium borate and polyvinylpyrrolidone (PVP) under a deoxidized condition are used to remove secondary metabolites and other interfering compounds. The pro-

cedure (4) was the following: 1 g of plant tissues was ground in liquid nitrogen and incubated in 5 mL extraction buffer containing 2 M NaCl, 25 mM EDTA, 200 mM Tris (pH 8.0), 20 mM sodium borate, 2% PVP (mol wt 40,000), 2% CTAB (w/v), 1% LSS (w/v), and 2% β -mercaptoethanol (v/v) at 65 °C in a water-bath shaker over 15 min with occasional shaking after each 5 min. Six milliliters of chloroform/isoamyl alcohol (24:1, v/v) were added into the mixture, and then mixed vigorously, followed by centrifuging at 14,000 rpm for 15 min at 4°C. The resulting aqueous solution (upper phase) was transferred into another tube containing 8 mL of water-saturated phenol/chloroform/isoamyl alcohol (25:24:1, v/v, pH 4.5). The sample was mixed vigorously and then centrifuged at 11,000 rpm for 10 min at 4°C; this step was repeated twice. The upper phase containing RNA was extracted with 8 mL chloroform/isoamyl alcohol (24:1, v/v) and then centrifuged at 11,000 rpm for 10 min at 4°C. The upper phase was transferred into a fresh centrifuge tube and 0.8 volumes (~6 mL) of 5M LiCl (Vetec) were added. The mixture was incubated at -20 °C overnight, followed by centrifuging at 14,000 rpm for 15 min at 4 °C. The pellet was rinsed three times with 5 mL of cold 75% ethanol. The air-dried pellet was ressuspended in 200 µL of TE and transferred into a microtube with 600 µL absolute ethanol and 10 µL 3 M NaAc (pH 5.2) at -20°C for 30 min. The samples were centrifuged at 14,000 rpm for 15 min at 4 °C. The pellet was air-dried and finally ressuspended in 50 µL of Milli-Q sterile water.

RNA purification and cDNA synthesis

After extraction, RNA samples were treated with 0.5 u/ug RNA of RNase-free DNase I (Invitrogen) for RT-PCR as follows: samples were incubated (10 µg RNA) at 37 °C for 15 min with 5U of the enzyme and 10X buffer (200 mM Tris HCl pH 8.3; 500 mM KCl; 25 mM MgCl₂). Milli-Q sterile water (450 μ L) and phenol (500 μ L) were added into the tube and mixed by inversion. Each sample was centrifuged at 10,000 rpm for 10 min and the supernatant was transferred into a new tube containing one volume of chloroform (~500 μ L). The sample was mixed by inversion and centrifuged as previously described. The supernatant was carefully transferred into a new tube and the RNA was precipitated with 0.1 volume of sodium acetate 3M and 2 volumes of 100% ethanol for 1 hour at -20°C. After centrifugation at 12,000 rpm for 20 min, the supernatant was discarded. The sample was rinsed with 500 μ L 70% ethanol and centrifuged at 10,000 rpm for 5 min. The supernatant was then discarded and the pellet was air-dried for 30 min. The RNA was ressuspended in 15 µL of Milli-Q sterile water. RNA integrity was assessed by electrophoresis on 1% nondenaturing agarose gel. The purity and yield of total RNA extracted from different tissues were measured by the NanoDrop, model ND-1000 spectrophotometer (NanoDrop Technologies; Wilmington, DE).

cDNAs were synthesized by adding 50 μ M of oligo (dT24V) and 10 mM of each deoxyribonucleoside 5'triphosphate (dNTPs) to 2 μ g of total RNA. This mixture was incubated at 65 °C for 5 min, and briefly chilled on ice. First Strand Buffer, 20 mM of dithiothreitol (DTT) and 200 units of Superscript III (Invitrogen) were added to the prior mixture and the total volume (20 $\mu L)$ was incubated at 50 °C for 1 h following fabricant instructions. Inactivation of the reverse transcriptase was done by incubating the mixture at 70 °C for 15 min.

Isolation of specific sequence of GAPDH gene from C. brasiliense

Thirty five sequences related to plant GAPDH were retrieved from GenBank databases and aligned using Clustal W multiple alignment. After the alignment, nine sequences were selected for primer design, all belonging to eurosid group, since C. brasiliense is an eurosid member. Degenerated primers were designed using SCPrimer program (JABADO et al., 2006) with the total number species, i.e. maximum degeneracy of 100. Design criteria for primers were as follows: 18-32 nt in length, Nearest Neighbor Tm 50–65°C (optimum 60°C) with sodium concentration of 50 mM, 40-60% GC, maximum allowed hairpins of 8 nucleotides, a maximum homopolymeric run of 4 nucleotides, no mismatches allowed in 3 pentamer, maximum of three mismatches to any targeted template and an amplicon size of 150-300 nucleotides. Primer pairs with melting temperatures within 3 °C of each other were selected. To visualize the position of degenerate primers, the GAPDH nucleotide sequences were aligned with the sequences forward and reverse of primers using the software Mega, version 3.0 (KUMAR et al., 2004).

The PCR was done in a total volume of 25 µL using 1 µL of cDNA from stem of C. brasiliense, 0.2 µM of each primer (F: 5' HGAYAAGGAMAARGCTGCTGC 3'; R: 5' ATCVACRGTDGGNACACGGA 3') 200 µM of each dNTP, 1X PCR Buffer (Fermentas), 0.25 units of Taq DNA polimerase (Fermentas). Reaction mixtures were incubated for 5 min at 95°C, followed by amplification 40 cycles of 30s at 95 °C, 45s at 55 °C and 120s at 72 °C; followed by an elongation step at 72 °C for 7 min, in a Mastercycler personal termocycler (Eppendorf). The PCR product generated with designed primer was cloned into pGEM-T Easy Vector and validated by sequencing. Sequencing reactions were performed for each clone (100 ngµL⁻¹) using the Perkin Elmer Biosystems Big Dye Terminator Sequence Reaction kit (Perkin Elmer, Foster City, CA) in the MegaBACE 1000 System (GE-Amersham Biosystem, Chalfont St. Giles, UK). BLAST analysis was performed to verify the similarity with other GAPDH.

To examine the structural relationship between *GAPDH* from *C. brasiliense* and *GAPCP-1* from *A. thaliana*, the genomic full length and CDS sequences of this gene from *A. thaliana* were retrieved from TAIR database and aligned with the *C. brasiliense* sequence cloned. We suggest the name *CbrGAPDH* for the sequence of *C. brasiliense GAPDH* cloned.

Reverse transcription polymerase chain reaction (RT-PCR)

Reaction mixtures contained 1 μ L of cDNA, 0.2 μ M of each primer, 200 μ M of each dNTP, 1X PCR Buffer (Fermentas), 0.25 units of Taq DNA polimerase (Fermentas), in a total volume of 25 μ L. Reaction mixtures were incu-

bated for 5 min at 94 °C, followed by 20, 25, 30 and 40 amplification cycles of 30s at 94 °C, 45s at 60 °C and 120s at 72 °C; followed by an elongation step at 72 °C for 7 min. PCR reactions were performed in a Mastercycler personal termocycler (Eppendorf). Two primers were designed to amplify a specific region of this gene: 5'-TTGTGGGTGTCAACGAGAAG-3' (forward) and 5'-CCCTCAACTATGCCAAACCT-3' (reverse). The amplification products were visualized on a 1% (w/v) agarose gel with ethidium bromide staining.

CbrGAPDH expression under water stress

In order to obtain plants under different conditions of watering we used *C. brasiliense* seeds from an Atlantic Forest located at Tiradentes-MG, Brazil. To accelerate the germination, we performed a slight groove at seeds epicarps prior sowing. Ten plants were well watered (control treatment) and ten plants had watering interrupted at 5 months of age (drought treatment). After 42 days, two leaves of each plant were water potential measured (Pressure Chamber PMS 1000 Instruments, Corvallis, OR, USA) and leaves from five plants in both conditions (control and drought) were collected, mixed and macerated in liquid nitrogen to RNA isolation (protocol



Figure 1. – Enzymatic manipulation of C. brasiliense DNA to assess quality. Total DNA leaf of C. brasiliense digested (5 µg) with PstI (1), EcoRI (2), HindIII (3) and HinfI (4), as compared to digestion of λ DNA (5 µg) with the same enzymes (1M) PstI, (2M) EcoRI, (3M) HindIII and (4M) HinfI. C. brasiliense un digested DNA (5 µg) is showed in lane (5).

3). All the subsequent steps to perform the expression analysis of *CbrGAPDH* followed the protocols described in the present work.

Results

DNA isolating

Our protocol of DNA extraction, using 300 mg of fresh material, results in yields of 107 (±3) µg for leaves, 42 (±16) µg for stems and 53 (±5) µg for roots. These yields were obtained after the adaptation of FERREIRA and GRATTAPAGLIA's protocol. The original protocol previously tested resulted in very poor yield and quality, what made it unsuitable for further applications in molecular biology (data not shown). We increased the concentration of PVP and 2- β -mercaptoethanol for 2% and 1%, respectively. The quality of the DNA was high (A_{260/280} ranging of 1.88 to 2.02). To further check the quality the DNA of *C. brasiliense*, it was digested with four distinct restriction enzymes. The agarose gel analysis indicated that the DNA is also suitable for enzymatic manipulation (*Fig. 1*).



Figure 2. – Gel eletrophoresis analysis of RNA (1 µg) from *C. brasiliense* extracted following the WANG et al. (2008) protocol. Leaf (lanes 1 and 2), stem (lanes 3 and 4) and root (lanes 5 and 6). The gel was visualized in 1% agarose gel and staining with ethidium bromide. M: λ DNA digested with *PstI*.

Table	1.	_	Yield	and	l p	urity	of	C.	bras	ilien	se	total	RNA	extr	acted	l from	diffe	erent	tissu	ıes	(leaf,	stem	and
root).	Da	ta	repre	esen	t m	lean	and	SI	E of 2	2 biol	og	ical r	eplica	tes.									

Protocol*	Tissue	RNA I	Yield	
		A _{260/280}	A _{260/230}	(µg/100mg)
(1) RNAeasy® (Qiagen)	Leaf	1.77 ± 0.06	0.77±0.02	0.65
	Stem	2.12±0.27	0.22±0.14	0.15
	Root	1.96 ± 0.44	$0.14{\pm}0.04$	0.18
(2) Trizol® (Invitrogen)	Leaf	1.55 ± 0.08	0.09±0.02	0.48
	Stem	1.67 ± 0.08	0.09 ± 0.04	0.46
	Root	1.76±0.13	$0.10{\pm}0.05$	0.55
(3) Invisorb (Invitek)	Leaf	2.09 ± 0.04	$1.84{\pm}0.07$	26.5
	Root	2.12 ± 0.01	1.82 ± 0.05	14.05
(4) Wang et al. 2008*	Leaf	$1.89{\pm}0.01$	1.89 ± 0.01	5.94
	Stem	2.07 ± 0.01	2.05±0.03	1.7
	Root	$2,02{\pm}0.04$	1.97 ± 0.02	1.43

RNA isolating

The RNA quality obtained by Trizol[®] (1) and RNeasy[®] Plant Mini Kit (2) procedures was unsatisfactory triggering problems even for agarose gel analysis, indicating excessive contamination. The low ratio $A_{260/280}$ of the RNA samples from Trizol[®] and RNeasy[®] Plant Mini Kit procedures suggest contamination by polysaccharides or phenols. The protocols (3) and (4) resulted in better yields of RNA (*Table 1*). The main change included at Wang's protocol (4) may have helped to achieve better yields was the extraction buffer incubation time which was prolonged for 15 minutes. This change resulted in clear supernatant and also a clear-cut peak at 260 nm that indicates RNA samples with low amounts of contaminating proteins, polysaccharides and polyphenolic compounds. Then, clearly visible band for 28S and 18S ribosomal RNA (rRNA) were observed on gel electrophoresis analysis indicating that such protocol preserved the RNA during the extraction without degradation (*Fig. 2*).

Gene Cloning and Sequencing

A 376-bp fragment of the *GAPDH* gene was amplified by RT-PCR using the degenerated primers which were designed based on alignment of nine homologues which were accessed at GenBank NCBI as follow: *Populus*

"#Populus #Calophyllum #Cbr_For #Cbr_Rev #Glycine #Cicer #Pisum #Medicago #Linum #Pyrus #Pyrus #Fragaria #Cucumis	ACT	GGA	GTT 	ттс :::: 	ACA -TC -H C C C C	GAC	AAG	GAC H T A	AAG	GCT	GCT	GCT	CAC	TTG C	AAG	GGT	GGT	GC? T A C C C T T	AAG	AAG	GTT AAG 	GTT A A A A A.C C	ATC T T G	TCC C.T T T T ATT A	GCC T A T TT 	CCA C C G.G C
#Populus #Calophyllum #Cbr_For #Cbr_Rev #Glycine #Pisum #Medicago #Linum #Pyrus #Fragaria #Fragaria	AGC	AAA G G G	GAT	G . AT 	-CA G C T G T C	CCT A c c c c	ATG	TTT 	GTT	GTG T T T T T T T	GGT	GTA C T T T C C G	AAT c	GAG	AAG C.C T C.T C.C	CAG G.A G.A G.A G.A G.A G.A G.A G.A G.A	TAC	ACT .AG .AG .AG .AG .AG .AG .AG	CCA AA. T.T	GAT G G G G G G	стт т.с	GAC A TG.	ATT	GTT A A.C C	TCC T T T	AAT
#Populus #Calophyllum #Cbr_For #Cbr_Rev #Glycine #Cicer #Pisum #Medicago #Linum #Pyrus #Fragaria #Fragaria	GCT	AGC T T T T T T	TGC	ACT	ACC	AAC	TGT C C C C C C	CTT 	GCC	CCCC	TTG C.C C.T C.T C.T C.T C.T C.T	GCC T T T G	AAG	GTT	ATC T T T T	CAT A A A A A A A	GAC T T	AGG A C.A A	 	GGA C C C C T	ATT A c c c	GTT G	GAG	GGT C 	CTT C T.G T.G C	ATG
#Populus #Calophyllum #Cbr_Ror #Cbr_Rev #Gicen #Pisum #Medicago #Linum #Fragaria #Fragaria	ACT c c c c	ACA C T T C C	GTT c c c c	CAC	GCG T.C T.C T.C T.C T.C T.C T.C	ATT c c c c c c	ACT	GCC T 	ACT T.C 	CAG	AAA G G G G	AC-	TGT	TGA G 	TGG	TCC A G G A A	ATC C T 	AAT .GC GC GC G G TTC .GC	GAA 	GGA A A A A A A	СТБ Т т	GAG	AGG G G G G	TGG G C	AAG	GGC A A A A A T A
#Populus #Calophyllum #Cbr_For #Cbr_Rev #Gicen #Fisum #Medicago #Linum #Fragaria #Fragaria	төс А	ттс с с с	CTT A A A A A A A	CAA T T T	TAT C C C C C C C	CAT T	тсс	TAG C C C C	CAG	CAC	тGG с 	GGC A A A A A A	TGC	CAA T A	GGC	т <u>ст</u>	TGG C A C C		GGT A A A A A A	TCT G G G A	GCC T T T T T T	AGC T.T T.T T.T T.A T.A T	ACT TT. GT. T. T. T. T. T.	TAA G G G G G G G.	тсс с с	AAA C G
<pre>#Populus #Calophyllum #Cbr_For #Cbr_Rev #Glycine #Cicer #Pisum #Medicago #Linum #Pyrus #Fragaria #Cucumis</pre>	ATT	GAC	TGG C C G C	AAT	GTC	 A A A A T	CCG	TGT A 	GCC ? C T C C T A	TAC A A A C	CGT T C T T	GGA T C T C T T	Ţ Ţ													

Figure 3. – Placement of phylogenetic informative indels in the aligned sequences from representatives taxa. Sequences correspond to the nine putative orthologues of CbrGAPDH (Calophyllum) aligned using CLUSTAL W. Dashes represent alignment gaps; dots indicate the same nucleotide as the reference Populus (DQ173770.1). The black boxes indicate the primers sequences: Cbr_For indicates the forward sequence and Cbr_Rev indicate the reverse sequence of degenerate primers.



Figure 4. – Representation of putative *C. brasiliense GAPDH* gene (*CbrGAPDH*) organization based on the comparison with *GAPCP-1* from *A. thaliana* by alignment. The black boxes represent the exons and the continuous line represents the introns. The numbers at *C. brasiliense* scheme are positions related to the *A. thaliana* full length genomic sequence (size = 3,333 bp).

maximowiczii \times Populus nigra (DQ173770), Glycine max (EU741646.1), Cicer arietinum (AJ010224.1), fulvum (EU269889.1), Medicago Pisum sativa (EF526095.1), Linum usitatissimum (AJ623267.1), Pyrus pyrifolia var. culta (AB266450.1), Fragaria \times ananassa (AB363963), Cucumis melo (AB033600.1) (Fig. 3). We selected these sequences according to the classification of species within eurosid group. The sequence analysis showed that the deduced amino acid sequence of C. brasiliense has high similarity to other GAPDHs reported. Among the GAPDH in GenBank, Nicotiana attenuata DQ682459.1 and Nicotiana langs $dorffii \times Nicotiana \ sanderae \ EF636821.1$ has the highest identities (85%) when compared as C. brasiliense. Putative conserved domains from NADB Rossman and Gp dh_C superfamilies were detected on *C. brasiliense* GAPDH protein sequence (data not shown). The structure of GAPDH gene from C. brasiliense was compared to the GLYCERALDEHYDE-3-PHOSPHATE DEHY-DROGENASE OF PLASTID 1 gene from Arabidopsis thaliana (At1G79530.1) available at TAIR Database. We aligned the C. brasiliense sequence obtained in this work with both full genomic and CDS sequences from Arabidopsis in order to visualize the exons and introns regions of this gene. The strategy was the alignment of three sequences (two from Arabidopsis plus one from C. brasiliense), which showed correspondence between exons of C. brasiliense and Arabidopsis (Fig. 4).

Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR reaction was performed using *GAPDH* primers to check the RNA quality for enzymatic manipulation and the potential of this gene as housekeeping in gene expression analysis. Transcripts of the expected size were obtained demonstrating that the protocols used were suitable for gene expression analyses. PCR products were observed from 30 cycles when the cDNA was the template for the three plant tissues (*Fig. 5*). The



Figure 5. – RT-PCR expression analysis of *CbrGAPDH* gene in leaves, stems and roots of *C. brasiliense*. Genomic DNA was used as PCR control.

different size between the products of amplification from DNA and cDNA is result of intron region in the *C. brasiliense* genomic DNA as expected by the comparative analysis with Arabidopsis gene structure (*Fig. 4*). Primer pairs that differentiate between genomic and cDNA are highly recommended for RT-PCR expression studies because can indicate genomic DNA contamination in cDNA preparations.

The expression levels of *GAPDH* from *C. brasiliense* (*CbrGAPDH*) were comparable among samples of the distinct tissues tested, could provide evidence that this gene can be a good reference to RT-PCR analysis. In order to provide further evidence of the gene expression stability of *CbrGAPDH*, we compare it expression in well watered plants ($\Psi = -1.04 \pm 1.5$ MPa; n = 10) and plants which were not watered during 42 days ($\Psi = -5.34 \pm 0.09$ MPa; n = 10). The drastic effects of the drought stress in the development of the *C. brasiliense* root system (*Fig. 6b* and *c*) did not result in differences in *CbrGAPDH* gene expression (*Fig. 6a*).







Figure 6. – Expression analysis of *CbrGAPDH* by RT-PCR in roots watered and root under drought stress (47 days after stop watering). 1Kb ladder (Invitrogen) was loaded on the left lane (a); Root development of *C. brasiliense* under regular watering (b) and under drought stress (c).

Discussion

The modifications implemented in this work for the DNA isolation to reduce the effects of oxidation with phenolic compounds and to protect the DNA from peroxidases and poliphenolidases (FERREIRA and GRATTA-PAGLIA, 1995) contributed for the *C. brasiliense* DNA integrity. Since the high NaCl concentration of the buffers helps to remove polysaccharides (CHANG *et al.*, 1993), we used NaCl 1.4 M on CTAB precipitation buffer instead of EDTA/Tris-HCl, employed in the FERREIRA and GRATTAPAGLIA's protocol. In summary, the modifications described in here were essential to allow isolation of gold-standard quality genomic DNA for enzymatic manipulation from *C. brasiliense* tissue samples.

The protocols Trizol® (1) and RNeasy® Plant Mini Kit (2) have been used successfully to isolate RNA from leaf tissue in several plants such as Arabidopsis (ALVES-FERREIRA et al., 2007), tobacco (MEISEL and LAM, 1996) and maize (DAS et al., 1990). Since most of the successful plant RNA isolation procedures use these two procedures, we decided to test them in C. brasiliense. However, these two traditional procedures resulted in poor yields and low quality RNA for all tissues tested on C. brasiliense. Inadequate results were also achieved with commercially available extraction kits, such as RNeasy® and Trizol®, to RNA extraction of medicinal plants that are rich in secondary metabolites (GHANGAL et al., 2009). Problems with RNA or DNA extraction of wild plants or wood plants are due to impurities such as terpenes, polyphenolics, and polysaccharides, which are often abundant in these plants and coextract with DNA or RNA. As a consequence, many tree or wild species require more complex extraction methods than do annual or crop plants (SHEPHERD et al., 2002). We suggest, for RNA isolation of C. brasiliense, the Invisorb® Spin Plant RNA Mini Kit (Invitek) or the method described here adapted from WANG et al. (2008). The main difference between the WANG's method and our protocol is the extended period in extraction buffer, which greatly increased the suspension of the tissue helping the RNA extraction. However, this procedure is time consuming and, all together, the procedure requires a large quantity of start material. The Invisorb (Invitek) method (3) combines the advantages of being fast, easily applicable to different tissues. Although, it can be expensive if a large amount of samples need to be prepared.

The strategy for cloning genes by the employment of degenerated oligos has been proved reliable and promising for exotic species with poor genomic information. However, the degeneracy parameter in the oligonucleotide design and the optimization at PCR cycles are critical for success (JABADO *et al.*, 2006; LÖFFERT *et al.*, 1998). The method described here was successful to isolation of the first cloned gene of *C. brasiliense*, the *CbrGAPDH*. The identification of the *CbrGAPDH* gene will have important applications in quantifying of expression levels of genes selected from functional genomics studies in *C. brasiliense*. The *GAPDH* gene can be also used as reference gene to perform quantitative real-time RT-PCR, a powerful tool to investigate relevant changes in gene expression in plants (CRUZ *et al.*,

2009; ARTICO *et al.*, 2010). However, it is always important to check the potential of CbrGAPDH as reference gene when a new experimental condition is tested.

Our results showed for the first time the successful application and adaptation of protocols to extract RNA and DNA from the rain forest wood plant *C. brasiliense* and the isolation of the *CbrGAPDH* gene, a reference gene for expression analysis. The development of these tools is the first step in the characterization of *C. brasiliense* by molecular genetics techniques. Therefore, it will be very useful for those interested to employ it as a model for ecogenomic of rain forest trees. Moreover, this work illustrated the steps necessary for building the essential framework for molecular genetics research in exotic plants with poor genomic information.

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